

genetic or secondary cause of iron accumulation or with relapse of leukemia. A possible explanation for this phenomenon could be increased intestinal iron absorption caused by chemotherapy-induced mucosal damage.

Our data confirm that there is not a clear-cut association between HH gene mutations and iron overload, in AML patients. However, as a significant percentage of long-surviving AML patients develop iron overload which then persists for several years after transfusions, the follow-up of these patients should include iron status measurement in order to intervene to prevent the development of complications.

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Antioxidant enzyme expression in myelodysplastic and acute myeloid leukemia bone marrow: further evidence of a pathogenetic role for oxidative stress?

We studied the expression of four antioxidant enzymes in normal, myelodysplastic (MDS) and acute myeloid leukemia (AML) bone marrow cells. Enzyme expression in normal marrow differed with lineage and cellular maturation. Expression was most frequently increased in MDS/AML granulocytes, but less so in CD34⁺ cells, suggesting that oxidative stress may select surviving progenitor cells for augmented antioxidant defence.

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Indirect evidence suggests a role for oxidant DNA damage in the pathogenesis of myelodysplasia.¹ An elevated plasma concentration of the lipid peroxidation product malondialdehyde² and the *in vitro* stimulation of MDS progenitor cells by thiol antioxidants such as amifostine,³ thioredoxin and N-acetyl cysteine⁴ provide some support for this.

We quantified expression of the antioxidant enzymes glutathione peroxidase 1 (GPX1), manganese superoxide dismutase (MnSOD), catalase (CAT) and the rate-limiting enzyme for glutathione synthesis, γ -glutamyl cysteine synthetase heavy sub unit (GCS), in bone marrow from healthy individuals (n=12), and from patients with MDS and AML [RA/RARS (n=21), RAEB (n=14), CMML (n=9), and AML/RAEBt (n=18)].

Bone marrow mononuclear cells (MNC) were isolated by density gradient centrifugation. Granulocytes were obtained as a high-density pellet, followed by ammonium chloride red cell lysis (apoptosis >2% in 3/25 and granulocyte/band form >70% in 20/25). CD34⁺/-, CD8⁺/- and glycophorin A (GPA)⁺/- cells were separated from MNC by magnetic labeling. Enzyme expression was quantified by real-time polymerase chain reaction (ABI 7700). TaqMan[™] primer and probe sequences and locations are listed in Table 1. 18S rRNA was used as reference RNA for quantification of target gene expression. Expression Ct value was first normalized for RNA concentration against an 18S ribosomal RNA internal control. This normalized expression ratio was then standardized for each plate relative to the normalized expression of an internal standard (K562 cell line for MnSOD, catalase, GCS, and HEL cell line for GPX1) and expressed as a standardized expression ratio (normalized

Table 1. Primer and probe sequences/genomic location.

Enzyme	Primer/probe	Location	Sequence
GPX1	Forward	Exon 1-2	5'-gcttccctgcaaccagtt-3'
	Reverse		5'-gcacttctcgaagagcatgaagt-3'
	Probe		5'-aatctcttcttcttggcgttctctctgatg-3'
MnSOD	Forward	Exon 2-3	5'-tgaacgtcaccaggagaagtagc-3'
	Reverse		5'-tgatagaccaccattgaac-3'
	Probe		5'-tgggctgaacatctccttggcca-3'
CAT	Forward	Exon 1-2	5'-cagcgaccagatgcagca-3'
	Reverse		5'-agtgaaaaccacatctgaacaag-3'
	Probe		5'-cgcagaagctgatgtctgaccactg-3'
GCS	Forward	Exon 1-2	5'-ttgcaggaaggcattgatca-3'
	Reverse		5'-agcatcatccagggtattttctctt-3'
	Probe		5'-ctggccagcatgttctcatctctt-3'

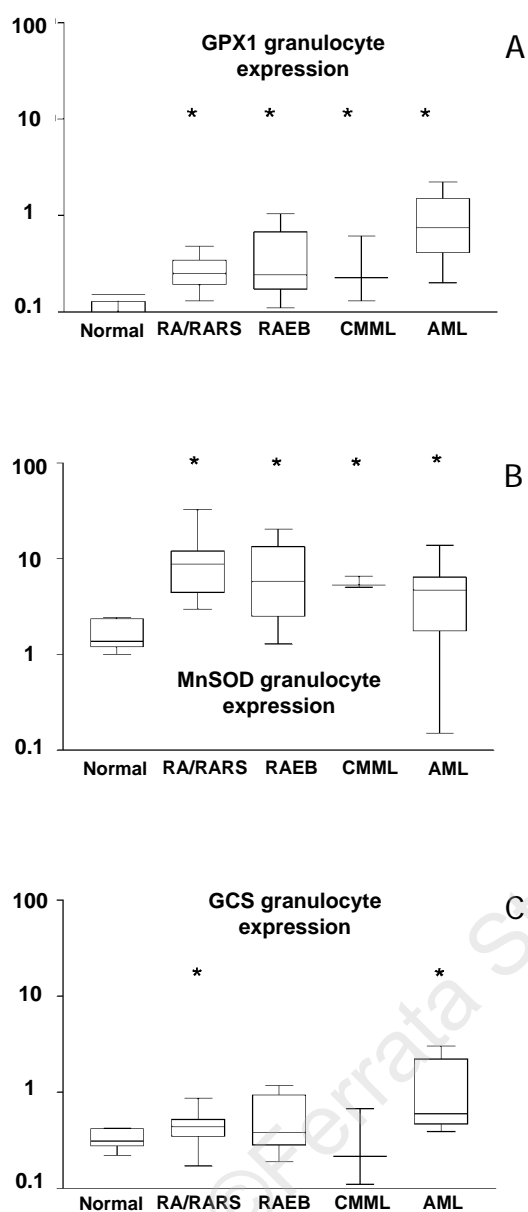


Figure 1. Expression of a) GPX1, b) MnSOD and c) GCS in granulocytes from MDS and AML bone marrow, with that in normal bone marrow for comparison. Enzyme expression is presented as the *standardized expression ratio* (y-axis), that is first normalized to the expression of a housekeeping gene, and then standardized to enzyme expression of a cell line (see text for details). Box-and-whiskers plots show upper and lower quartiles (*box*), median (*line within box*) and range (*whiskers*). * $p < 0.05$ for difference from normal.

expression ratio for test/normalized value for cell line)(Figure 1, y-axis). Kruskal-Wallis analysis was used to establish both inter-lineage differences in normal subjects and inter-FAB type differences for patients. Mann-Whitney analysis was used to compare differences between two individual FAB groups for the same enzyme.

Expression differed between lineages in normal marrow for all enzymes ($p < 0.003$ for each). Within each lineage, inter-sub-

ject expression variation was relatively limited (< 3 -fold difference). Between lineages, the highest fold variation in enzyme expression was seen for GCS (GPA⁺ cells ~ 20 -fold greater expression than granulocytes) and GPX1 (GPA⁺ and CD34⁺ cells ~ 10 -fold greater expression than granulocytes). Differences in enzyme expression between progenitor-enriched CD34⁺ cells and the more heterogeneous mononuclear cell population were not marked. In contrast, granulocyte expression of GPX1 and GCS was considerably lower, and of MnSOD considerably higher than for mononuclear cell/progenitor populations. Thus, antioxidant enzyme expression is considerably influenced by myeloid maturation stage.

Significant differences between MDS/AML and normal bone marrow enzyme expression were noted for GPX1, MnSOD and GCS, with the largest differences being found in granulocyte fractions. Expression of only 1/4 enzymes (GPX1) was increased in MDS CD34⁺ cells and then only in low-risk patients ($p < 0.0134$: RA/RARS and RAEB). Expression of 2/4 antioxidant enzymes was increased in MDS mononuclear cells. GPX1 was increased in all FAB types except CMML ($p < 0.0001$), while MnSOD was increased in all FAB types except AML/RAEBt ($p < 0.0218$). In quantitative terms, increased mononuclear cell expression in MDS compared with normal, was modest (2-fold) for both enzymes. Expression of 3/4 enzymes was increased in MDS granulocytes (Figure 1). Granulocyte GPX1 expression was increased in all FAB types ($p = 0.0001$), as was MnSOD expression ($p = 0.0083$). GCS expression was increased in RA/RARS and to a greater extent in AML ($p = 0.0115$). Upregulation of enzyme expression was quantitatively greater in granulocytes than mononuclear cells, with GPX1 expression increased 3-fold (RA/RARS) to 9-fold (AML/RAEBt), MnSOD expression was increased 3-fold (AML/RAEBt) to 6-fold (RA/RARS), and GCS expression only 1.5-fold (RA/RARS) to 2-fold (AML/RAEBt). Antioxidant enzyme expression was not a useful prognostic parameter as indicated by a lack of correlation with IPSS score or survival. Granulocyte GPX1 expression was consistently lower in remission AML samples than in presentation samples with-in the same patient (*not shown*).

Our observations may reflect induction of antioxidant enzyme expression or selection of high-expressing clones. Our data support a model for the role of oxidative stress in the ineffective hematopoiesis of MDS, analogous to that proposed to explain clonal expansion of a dysplastic clone in Fanconi's anemia.⁵ We suggest that the CD34⁺ compartment in MDS may be under oxidative stress. Many CD34⁺ progenitors will fail to survive because of inadequate stress defence, a model supported by recent gene-expression profiling.⁶ Clonal selection of resistant clones may then be promoted, which in turn gives rise to mature progeny with augmented antioxidant capacity.

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Effects of the MEK inhibitor CI-1040 (PD 184352) on progenitor growth from normal and myelodysplastic marrow

Previous studies demonstrated that ~50% of acute myelogenous leukemia samples exhibit activation of the mitogen-activated protein kinase (MAPK) pathway¹⁻³ and growth inhibition by MEK inhibitors.³ To extend these observations, we assessed MAPK pathway activation and effects of CI-1040 in normal and myelodysplastic (MDS) marrow.

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Activation of the mitogen-activated protein kinase (MAPK) pathway, as manifested by phosphorylation of extracellular signal regulated kinases (ERK) 1 and 2 on a specific Thr-X-Tyr motif, is usually assessed by immunoblotting.

Because normal and myeloblastic (MDS) marrow contain mixed cell populations, immunohistochemistry was utilized to assess cell type-specific pathway activation. Under Institutional Review Board-approved protocols, B5-fixed, paraffin-embedded marrows from 14 MDS patients (3 RA, 5 RAEB, 3 RCMD, 3 CMML) and 4 controls were treated with 3% H₂O₂ to inhibit peroxidases, subjected to heat-induced antigen retrieval, and stained with rabbit anti-phospho-ERK1/2 (anti-ACTIVE™ p44/42 MAPK; Promega, Madison, WI, USA) followed by biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin. Fixed, paraffin-embedded HL-60 leukemia cells and normal human tonsil served as positive and negative controls, respectively. This analysis demonstrated MAPK activation in all MDS marrows examined. As illustrated in Figure 1A, staining was strongest in blast cells and myeloid progenitors, intermediate in erythroid cells, and weakest in megakaryocytes. The staining strength and pattern did not differ among MDS subtypes. In normal marrow, a similar pattern was observed, although the staining intensity was lower (Figure 1B).

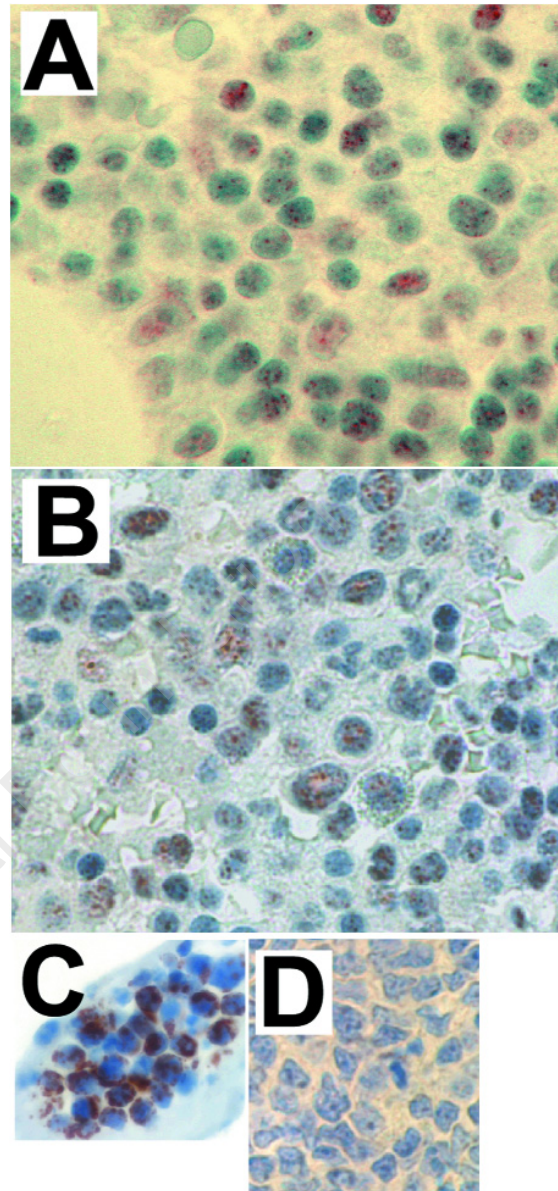


Figure 1. Immunohistochemical staining for dually phosphorylated ERK1/2 in MDS and normal marrow. A, marrow biopsy specimen from a patient with RAEB. B, marrow biopsy specimen from a normal control. C, phospho-ERK staining in HL-60 cells, which are known to have MAPK activation.^{3,9} D, lack of phospho-ERK staining in follicles from normal human tonsil. All panels were counterstained with hematoxylin and photographed at 400X magnification.

In view of these results, the effect of the MEK inhibitor CI-1040⁴ on growth of erythroid and myeloid progenitors *in vitro* was examined. Fresh marrow from 12 MDS patients, 14 patients with cytopenias but no evidence of MDS, and 3 normal patients undergoing hip arthroplasty, as well as blood from 5 normal volunteers was fractionated on Ficoll-Hypaque gradients. Mononuclear cells (6×10^5 /aliquot) were plated in 3 mL Methocult™ methylcellulose medium (StemCell Technologies; Vancouver, BC, USA) containing diluent or 10 μ mol/L CI-